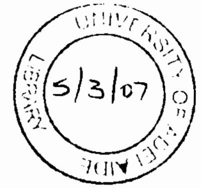
09PH
P3933



EXPRESSION AND FUNCTION OF GENES IDENTIFYING PLURIPOTENT CELL SUB-POPULATIONS IN THE EARLY MOUSE EMBRYO

A Thesis Submitted To The University Of
Adelaide For The Degree Of Doctor Of Philosophy

by

Tricia Ann Pelton, B Sc. (Hons.)

Department Of Biochemistry
University Of Adelaide
Adelaide, South Australia

July, 2000

TABLE OF CONTENTS

SUMMARY ... i
STATEMENT ... iii
ACKNOWLEDGEMENTS ... iv

CHAPTER 1: GENERAL INTRODUCTION

1.1 MAMMALIAN DEVELOPMENT ... 1
1.2 PLURIPOTENT CELLS AND MAMMALIAN EMBRYOGENESIS ... 1
1.3 MOUSE DEVELOPMENT ... 2
1.3.1 Pre-implantation Development ... 2
1.3.1.1 Fertilisation and first cleavage divisions ... 2
1.3.1.2 Compaction and cell polarisation ... 3
1.3.1.3 Blastocyst formation ... 4
1.3.2 Peri-implantation Development ... 4
1.3.2.1 Blastocyst implantation ... 4
1.3.2.2 Cellular changes in the embryo ... 5
(i) Cellular changes within the trophectoderm ... 6
(ii) Primitive endoderm differentiation ... 6
(iii) Formation of the egg cylinder ... 7
1.3.3 Gastrulation and Axis Patterning ... 8
1.4 EVIDENCE FOR TRANSIENT PLURIPOTENT CELL SUB-POPULATIONS IN THE EARLY MOUSE EMBRYO ... 11
1.4.1 Developmental Potential of Pluripotent Cells ... 11
1.4.1.1 Loss of totipotence ... 11
1.4.1.2 Potency of ICM to form primitive endoderm ... 12
1.4.1.3 Primitive ectoderm developmental potential ... 13
1.4.1.4 Summary ... 14
1.4.2 Gene Expression in Pluripotent Cells ... 14
1.4.2.1 Differential gene expression during pre and early post-implantation development 15
1.4.2.2 Regionalised gene expression within pre-gastrulation primitive ectoderm ... 16
1.4.2.3 Summary ... 18
1.4.3 Complex Regulation of Pluripotent Cell Populations during Peri-implantation Development ... 18
1.4.3.1 Pluripotent cell maintenance ... 18
1.4.3.2 Paracrine signaling between ICM and extra-embryonic cells ... 19
1.4.3.3 Pluripotent cell proliferation ... 20
(i) Early proliferative pluripotent cell defects ... 21
(ii) Proliferation defects in pluripotent cells following egg cylinder formation ... 22
(iii) Summary ... 22

1.4.3.4 Primitive ectoderm formation and maintenance; an instructive role for visceral endoderm 23
1.4.3.5 Summary 25
1.5 *IN VITRO* MODELS OF EARLY EMBRYOGENESIS 25
1.5.1 Embryonal Carcinoma Cells 26
1.5.2 Isolation of Embryonic Stem Cells 27
1.5.3 Differentiation of ES Cells *in vitro* 29
1.5.3.1 Spontaneous and chemical induction of ES cell differentiation 29
1.5.3.2 Embryoid body differentiation 30
1.5.3.3 Lineage specific ES cell differentiation 31
1.6 A SYSTEM FOR THE GENERATION OF PLURIPOTENT CELL MARKER GENES ... 33
1.6.1 Differential Message Display PCR 34
1.6.2 dd-PCR of ES and EPL Cell RNA 35
1.6.3 Sequence of *L17*, *Psc1* and *K7* Markers 36
1.7 AIMS AND APPROACH 37

CHAPTER 2: MATERIALS AND METHODS

2.1 ABBREVIATIONS 38
2.2 MOLECULAR MATERIALS 40
2.2.1 Chemicals and Reagents 40
2.2.2 Radiochemicals 41
2.2.3 Kits 41
2.2.4 Enzymes 41
2.2.5 Buffers and Solutions 42
2.2.6 Plasmids 44
2.2.6.1 Cloning Vectors 44
2.2.6.2 Cloned DNA Sequences 45
2.2.7 Oligonucleotides 45
2.2.7.1 General sequencing primers 45
2.2.7.2 Gene specific sequencing primers 46
2.2.7.3 PCR Primers 46
2.2.8 Antibodies 47
2.2.9 Bacterial strains and Media 47
2.2.10 Yeast Strains and Media 48
2.2.11 DNA Markers 49
2.2.12 Protein markers 49
2.3 MOLECULAR METHODS 49
2.3.1 Rapid Small Scale Preparation of DNA (Mini-prep) 49
2.3.2 Rapid Mid Scale Preparation of DNA (Midi-prep) 49
2.3.3 Large Scale Plasmid Preparation for Transient Transfection Analysis 50

2.3.4 Restriction Endonuclease Digestion of DNA....51
2.3.5 Agarose Gel Electrophoresis....51
2.3.6 Plasmid DNA Southern Blot....52
2.3.7 Purification of Linear DNA Fragments....52
2.3.8 Blunting DNA Fragments....52
2.3.9 Preparation of Vector DNA....53
2.3.10 DNA Ligation Reactions....53
2.3.11 Preparation of Competent Cells....53
2.3.12 Transformation of Competent Bacterial Cells....53
2.3.13 Sequencing of Plasmid DNA....54
2.3.14 Library Screening....54
2.3.14.1 Preparation of plating bacteria....54
2.3.14.2 Library plating....55
2.3.14.3 Plaque lifts....55
2.3.14.4 High Titre Stock Production....55
2.3.15 Lambda Zapping....56
2.3.16 Reverse Transcription....56
2.3.17 PCR....56
2.3.18 Isolation of Cytoplasmic RNA from Cultured Cells....56
2.3.19 Isolation of Polyadenylated RNA....57
2.3.20 Isolation of RNA from Mouse Tissues....57
2.3.21 Ribonuclease Protection Analysis....58
2.3.21.1 Riboprobe templates and probe synthesis....58
2.3.21.2 RNAse protection assay....58
2.3.22 Northern Blot Analysis....59
2.3.23 Synthesis of Radioactive DNA Probes....59
2.3.24 Hybridisation of Radioactive DNA Probes to Nylon and Nitrocellulose Filters....60
2.3.25 Phosphorimager Analysis and Image Manipulation....60
2.3.26 Transformation of Yeast Strains....61
2.3.27 Cos-1 Cell Lysis....61
2.3.28 Immunoprecipitation....61
2.3.29 SDS-PAGE Analysis....62
2.3.30 Western Analysis....62
2.3.31 Immunofluorescence....63
2.3.32 Flow Cytometric Analysis of Transiently Transfected Cos-1 Cells....64
2.3.32.1 Flow cytometric analysis of the entire population of transfected Cos-1 cells....64
2.3.32.2 Flow cytometric analysis of transfected Cos-1 cells within the population....64
2.3.33 Sequencing Software and Database Searches....65
2.3.34 Containment Facilities....66
2.4 EMBRYOLOGICAL METHODS....66

2.4.1 Mouse Strains and Matings 66
2.4.2 Embryo Processing Baskets 66
2.4.3 Embryo Isolation and Dissection 66
2.4.4 Embryo Fixation and Dehydration 67
2.4.5 Digoxygenin Labelled RNA Probes 67
2.4.5.1 Preparation of templates 67
2.4.5.2 DIG labelled riboprobe synthesis 68
2.4.6 Embryonic *in situ* Hybridisation 68
2.4.7 Mouse Embryo Powder 70
2.4.8 Antibody Preblocking 70
2.4.9 Animal Manipulations 70
2.5 POLYCLONAL ANTIBODY GENERATION 70
2.5.1 Generation of GST-K7 peptide constructs 70
2.5.2 Preparation of Competent BL21 Cells 71
2.5.3 Small Scale GST-fusion Protein Induction 71
2.5.4 Large Scale GST Fusion Protein Induction 71
2.5.5 Preparation of GST-fusion Protein for Immunisation 72
2.5.6 Immunisation Regime 72
2.6 TISSUE CULTURE MATERIALS AND METHODS 72
2.6.1 Chemicals and Reagents 72
2.6.2 Materials 73
2.6.3 Cell Lines 73
2.6.4 Solutions 74
2.6.5 Media 74
2.6.6 Maintenance of Cells Lines 75
2.6.7 Transient Transfection of Cos-1 Cells 76

CHAPTER 3: TRANSIENT PLURIPOTENT CELL SUB-POPULATIONS EXIST DURING PERI-IMPLANTATION MOUSE DEVELOPMENT

3.1 INTRODUCTION 78
3.2 *IN VIVO* EXPRESSION ANALYSIS OF MARKER GENES DURING EARLY MOUSE EMBRYOGENESIS 79
3.2.1 Establishment of Morphologically Distinguishable Stages During Peri-implantation Development 79
3.2.2 *In situ* Hybridisation Analysis of the ICM to Primitive Ectoderm Transition using Established Marker Genes, *Oct4*, *Rex1* and *Fgf5* 80
3.2.2.1 Oct4 Expression In vivo during Pre- and Early Post-implantation Mouse Development 81
3.2.2.2 Rex1 Expression In vivo during Pre- and Early Post-implantation Mouse Development 82

3.2.2.3 Fgf5 Expression In vivo during Pre- and Early Post-implantation Mouse Development 82
3.2.3 *In situ* hybridisation Analysis of the ICM to Primitive Ectoderm Transition *In vivo* using Marker Genes Identified by dd-PCR Analysis of ES cells and EPL cells 83
3.2.3.1 L17 Expression In vivo during Pre- and Early Post-implantation Mouse Development 83
3.2.3.2 Psc1 Expression In vivo during Pre- and Early Post-implantation Mouse Development 83
3.2.3.3 K7 Expression In vivo during Pre- and Early Post-implantation Mouse Development 84
3.2.4 Summary 85
3.3 *IN VITRO* EXPRESSION ANALYSIS OF MARKER GENES DURING THE ES TO EPL CELL TRANSITION 85
3.4 DISCUSSION 87
3.4.1 Gene Expression Analysis Demonstrates the Existence of Transient Pluripotent Cell Sub-populations *In Vivo* 88
3.4.2 Pluripotent Cell Sub-populations Correlate with Embryological Events 91
3.4.3 Expression of Marker Genes in Non-Pluripotent Cells 92
3.4.4 Gene Expression Analysis Demonstrates the Existence of Transient Pluripotent Cell Sub-populations *In Vitro* During the ES to EPL Cell Transition 93
3.4.5 ES and EPL Cell Embryonic Equivalents 95
3.4.6 Markers for the Identification of Other Pluripotent Cell Populations 96
3.4.7 Nomenclature for Pluripotent Cell Sub-populations 97

CHAPTER 4: MOLECULAR ANALYSIS OF THE MARKER GENE, *K7*
4.1 INTRODUCTION 99
4.2 ISOLATION AND SEQUENCE ANALYSIS OF THE *K7* cDNA 100
4.2.1 Isolation of *K7* cDNA Clones 100
4.2.2 Sequence Analysis of the *K7* cDNA and Identification of a K7 Open Reading Frame 102
4.2.3 *K7* cDNA and Protein Sequence Comparisons 103
4.2.3.1 DNA sequence alignments 103
4.2.3.2 Protein sequence alignments 103
4.3 CHROMOSOME LOCALISATION OF K7 104
4.4 *K7* EXPRESSION IN FOETAL AND ADULT TISSUES 106
4.4.1 *K7* Expression During Gastrulation 106
4.4.2 *K7* Expression During Later Mouse Embryogenesis 106
4.4.3 *K7* Expression in Adult Tissues 107
4.5 DISCUSSION 108
4.5.1 The *K7* cDNA 108
4.5.2 Potential Function of *K7* in Development and Adult tissues 110

4.5.3 A Potential Cellular Function for *K7*.....111
4.5.3.1 Chromosome segregation in yeast.....112
4.5.3.2 The role of cut1 in sister chromatid separation in S. pombe.....112
4.5.3.3 ESP1 function in S. cerevisiae.....114
4.5.3.4 Sister chromatid separation in vertebrates.....115

CHAPTER 5: FUNCTIONAL ANALYSIS OF THE *K7* GENE
5.1 INTRODUCTION.....117
5.2 *K7* FUNCTION IN MAMMALIAN CELLS.....118
5.2.1 K7 Interacts with Mouse PTTG protein.....118
5.2.1.1 Cloning of mPTTG and construction of a mPTTG-FLAG containing mammalian expression vector.....118
5.2.1.2 Cloning of a full length K7 cDNA construct and pEGFP-K7 mammalian expression plasmid.....119
5.2.1.3 Immunoprecipitation analysis of K7 and mPTTG.....119
5.2.2 K7 Protein Localises to Centrosomes.....120
5.2.2.1 Sub-cellular localisation of K7.....120
5.2.2.2 Co-localisation of EGFP-K7 and γ-tubulin, a centrosomal marker.....121
5.2.3 mPTTG Protein Localisation.....122
5.2.4 Overexpression of mPTTG Blocks Cells in G2/M phase.....123
5.2.5 Analysis of K7 Overexpression on Cell Cycle Progression.....125
5.3 *ESP1* AND K7 C-TERMINAL SEQUENCES ARE NOT FUNCTIONALLY INTER-CHANGEABLE.....126
5.3.1 Rationale.....126
5.3.2 Substitution of Two C-terminal *K7* Sequences for Corresponding C-terminal Sequences of *ESP1* and Expression in S252 *ESP1*^{ts} Strain does not Rescue the Mutant Phenotype.....127
5.4 DISCUSSION.....129
5.4.1 Interaction between K7 and mPTTG may Negatively Regulate K7 Activity.....129
5.4.2 Analysis of K7 and mPTTG protein localisations.....130
5.4.3 A Role for K7/mPTTG during Early Mitosis in Mammals.....131
5.4.4 *K7* was unable to Induce Chromosome Segregation in Yeast.....132

CHAPTER 6: GENERAL DISCUSSION
6.1 INTRODUCTION.....134
6.2 PLURIPOTENT CELL SUB-POPULATIONS DURING EARLY MOUSE EMBRYOGENESIS.....134
6.2.1 Applications of Marker Genes to the Analysis of Pluripotent Cell Development.....135
6.2.1.1 Analysis of pluripotent cell development in the mouse.....135
(i) Functional analysis of pluripotent cell developmental progression *in vitro*.....135

(ii) Functional analysis of pluripotent cell development *in vivo* 137
(iii) A molecular approach to the identification of signaling networks that regulate pluripotent cell progression 138
6.2.1.2 Relationships between pluripotent cell populations in vivo and in vitro 139
6.3 ANALYSIS AND POTENTIAL FUNCTIONS OF THE *K7* GENE 140
6.3.1 Future Analysis of *K7* Function 142
6.3.1.1 Analysis of the sub-cellular localisation of K7 and mPTTG during the cell cycle 142
6.3.1.2 Identification of proteins that interact with K7 143
6.3.1.3 Determining the role of K7 in dissociation of proteins from chromatin in an in vitro assay system 144
6.3.1.4 Analysis of K7 function in pluripotent cells in vitro 145
6.3.1.5 Analysis of K7 expression in vivo 146
6.3.1.6 Analysis of K7 function in pluripotent cells in vivo 147

BIBLIOGRAPHY 149

SUMMARY

The coordinated regulation of pluripotent cell developmental progression during early mouse development is critical for formation of the three primary germ layers of the embryo. Accumulating evidence suggested the existence of transient pluripotent cell sub-populations in the early mouse embryo, which could not be recognised at the molecular level. Marker genes generated by dd-PCR analysis of the ES to EPL cell transition *in vitro*, a model of the ICM to primitive ectoderm transition *in vivo* (Rathjen *et al.*, 1999; Lake *et al.*, 2000), potentially provided a means to distinguish these transient pluripotent cell sub-populations.

Wholemount *in situ* hybridisation analysis of the reported marker genes, *Oct4*, *Rex1* and *Fgf5* (Haub and Goldfaub, 1991; Hébert *et al.*, 1991; Rogers *et al.*, 1991; Rosner *et al.*, 1990, Schöler *et al.*, 1990a and b) in conjunction with markers identified and isolated from the dd-PCR screen of ES and EPL cell RNA, *L17*, *Psc1* and *K7* (Schulz, 1996; S. Sharma, unpublished) provided a molecular description of pluripotent cell developmental progression during peri-implantation mouse development. Expression of *Rex1* and *L17* was observed in the ICM and early epiblast populations of the developing blastocyst, and downregulated at approximately 4.75 d.p.c.. *Psc1* expression was detected in the pluripotent cells of the developing blastocyst and epiblast bud, and downregulated following proamniotic cavitation at 5.25 d.p.c.. *K7* was not expressed in the early blastocyst, but was expressed by pluripotent cells between 4.75 and 5.25 d.p.c., following the formation of the late stage blastocyst to immediately prior to primitive ectoderm organisation into a pseudostratified epithelial sheet. *Fgf5* expression was not detected during blastocyst development, but was detected in the primitive ectoderm following proamniotic cavitation at 5.25 d.p.c.. Compilation of gene expression data indicated the existence of at least four distinct pluripotent cell sub-populations within the *Oct4*$^+$ pool. Comparison of pluripotent cell sub-populations with embryonic development indicated that the transient sub-populations correlated with embryonic events, including increased pluripotent cell proliferation, proamniotic cavitation and formation of a primitive ectoderm monolayer. Furthermore, gradual progression of pluripotent cells *in vivo* correlated closely with marker gene expression *in vitro* during the ES to EPL cell transition, validating the conversion of ES cells to EPL cells as an *in vitro* model of the ICM to primitive ectoderm transition and allowing more precise definition of ES and EPL cell embryonic equivalents.

K7 was selected for further analysis, based on its tight expression window during early development. cDNA clones spanning a 6.6 kb *K7* sequence were isolated and the complete *K7* cDNA was shown to be 79.3% identical to a human gene of unknown function, termed KIAA0165. This human gene was expressed in similar tissues to *K7*, including the testis, haematopoietic system and gastrointestinal tract, suggesting that these two genes were homologues. The C-terminal putative protein sequence of K7 and KIAA0165 demonstrated similarity to three fungal proteins, *cut1* (*S. pombe*), *ESP1* (*S. cerevisiae*) and *bimB* (*A. nidulans*). Sequence similarity of the conserved C-terminal region of K7 with Cut1 and Esp1 C-terminal protein sequence implicated K7 in the control of cell division in mammals. A mitotic role for K7 was consistent with the *in vivo* expression pattern of *K7* in the adult, which was confined to tissues containing rapidly proliferating cells for tissue and cellular regeneration. Functional investigation of K7 in mammalian cells using overexpression studies demonstrated that like KIAA0165, K7 interacted with a known cell cycle regulator encoded by the *pituitary transforming tumour gene (PTTG)*. Furthermore, overexpression of mPTTG in Cos-1 cells increased the proportion of cells in the G2/M phase of the cell cycle, consistent with a role for this complex in cell cycle progression through G2/M. K7 was also shown to localise to the centrosome, the microtubule organising centre from which the mitotic spindle emanates. Taken together these results suggested that the K7/mPTTG interaction shared similarities with the Cut1/Cut2 and Esp1/Pds1 complexes of budding yeast and fission yeast respectively, suggesting that aspects of the chromosome segregation mechanism may be conserved between yeast and mammals.